DOCKET NO: 245617US0

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF	:
HIDENOBU SENPUKU, ET AL.	: EXAMINER:
SERIAL NO: 10/714,852	:
FILED: NOVEMBER 18, 2003	: GROUP ART UNIT: 1634
FOR: METHOD FOR EXAMINING THE CARIES RISK	:

DECLARATION UNDER 37 C.F.R. 1.132

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ALEXANDRIA, VIRGINIA 22313

SIR:

I, Hidenobu Senpuku hereby declare:

1. I am employed by National Institute of Infectious Diseases as an researcher and have experience in the field of Microbiology and Immunology.

2. I am familiar with the specification of the above-identified patent application.

3. The following observations and experiments were carried out by me or under my direct supervision and control.

4. As discussed at line 13 of page 8 to line 8 of page 9 of the specification, the HLA genes group include hetero -dimers and have a pair of DRB*1 genotypes in class II.

5. In the attached table, in the top horizontal column and most left vertical column, genotypes of DRB1 in class II are listed. We have identified the genotypes of DRB1 in class II for 59 people (41 women and 28 men) and the identified pair of genotypes are marked in the Table. Where no positions are marked for certain genotypes this means that there is no identified pair of genotypes.

10. The undersigned declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believe to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Hidenobu Senpuku
Name

Hidenobu Senpuku
Signature

April 27, 2006
Date

Clin Exp Immunol 2004; 137:393–401 doi:10.1111/j.1365-2249.2004.02548.x

Role of peptide antigen for induction of inhibitory antibodies to *Streptococcus mutans* in the human oral cavity

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(Accepted for publication 20 May 2004)

SUMMARY

The alanine-rich repeating region (A-region) in the surface protein antigen (PAc) of *Streptococcus mutans* has received much attention as an antigenic component for vaccines against dental caries. The PAc (residue 361–386) peptide in the A-region possesses a multiple binding motif (L- -V-K- -A) to various HLA-DR molecules and a B-cell core epitope (- Y- - -L- -Y- - - -) that recognizes the inhibiting antibody to *S. mutans*. In the present study, we investigated the immunogenicity of the PAc (361–386) peptide in humans and regulators of induction of the anti-PAc (361–386) peptide IgA antibody (aPPA) in saliva. The PAc (361–386) peptide was confirmed as an ideal peptide antigen for induction of the inhibiting antibody to *S. mutans* in 151 healthy human subjects (36·6 ± 12·6 years old) by quantitative analyses of oral bacteria and ELISA, as the aPPA titre in human saliva decreased significantly in an age-dependent manner. Homozygous *DRB1*0405* and *1502*, and heterozygous *DRB1*0405/1502* showed a negative association with production of aPPA and tended to reduce the number of total streptococci in saliva. In contrast, the *DRB1*1501* allele was significantly correlated with a high level of induction of the antibodies, and also tended to reduce lactobacilli and mutans streptococci. Further, peptide immunogenicity was confirmed in NOD-SCID mice grafted with human peripheral blood mononuclear cells. Our results indicate that the interplay between regulators such as age, *DRB1* genotype, cytokines, and peptide immunogenicity may provide a potential means for developing a vaccine useful for the prevention of dental caries as well as their diagnosis.

Keywords NOD-SCID mice peptide *DRB1* genotype *Streptococcus mutans* dental caries

INTRODUCTION

Streptococcus mutans has been suggested to have an association with dental caries [1,2], and epidemiological surveys have shown that greater numbers of *S. mutans* in children are associated with a higher incidence of decayed, missing, and filled teeth (DMFT), i.e. fragment caries experiences [3–5]. The cell surface protein antigens of *S. mutans*, PAc [6], Ag I/II [7], PI [8], and B [9], function essentially for colonization of the bacterium on tooth surfaces and interact with the salivary pellicle that coats the dental enamel [10–12]. The alanine-rich repeating region (residue 219–464, A-region) of the PAc molecule is important for the interaction of *S mutans* with salivary film [13–15] with a strong immunogenicity in humans [16], and may be a candidate antigen for inducing the production of inhibiting antibodies against the adherence of *S. mutans* to tooth surfaces.

The A-region is composed of 3 long and 2 incomplete repeating sequences [6]. Each repeating sequence contains sequences homozygous to the amino acid sequence, ^{365}TYEAALKQYEADL^{377}, while PAc (365–377), an important region for the adherence of *S. mutans* to tooth surfaces [17,18], as well as T- and B-cell epitopes overlap [17,19]. Further, the epitope (YEA-L-QY) of the surface protein antigen (PAg) of *S. sobrinus* [20] and its core B-cell epitope (- Y- - -L- -Y- - - -) are essential sequences in the antigenic epitopes of the PAc protein that are recognized specifically by the antibody [21]. The antibodies reacting with the core B cell epitope inhibit competitively interaction of *S. mutans* to salivary components [17,18,21]. The overlapped PAc (370–386) peptide to PAc (361–377) peptide includes a multiple binding motif (L- -V-K- -A) that reacts with HLA-*DRB1*0802*, **1101*, **1401*, and **1405* [22,23], and is also recognized in the A-region. Therefore, the coupled PAc (361–386)

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peptide from residues 361–377 and 370–386 may be a minimum antigen of PAc that induces the inhibiting antibodies for adherence of *S. mutans* to the tooth surfaces coated by salivary components in humans.

Salivary immunoglobulin A (IgA) reacts with oral streptococci and other bacteria, and is considered an important factor for host defense against infection [24]. These important functions of IgA have focused interest on the development of mucosal vaccines [25,26], as well as its possible therapeutic use in treatment of infection [27–29]. In addition, saliva levels of the IgA antibody are associated with caries protection, because negative correlations between the IgA antibody and caries formations have been found [30–32], and salivary IgA antibodies have been reported to play an important role against *S. mutans* for the prevention of dental caries through bacteriostasis [30,31]. The human leucocyte antigen (HLA) is coded by the major histocompatibility complex (MHC) and also plays an important role in controlling the production of antibodies in saliva [33,34], as the production of salivary IgA antibodies is influenced by HLA molecules on the immune cells [33–35]. In addition, the association between the HLA allele and susceptibility to colonization by *S. mutans* or production of the salivary IgA antibody has attracted extensive interest in regards to the development of a dental caries vaccine. To investigate whether the PAc (361–386) peptide has a function as an effective antigen regarding the induction of human antibodies influenced by the HLA class II polymorphism in human saliva, we examined anti-PAc (361–386) peptide antibody titres in human subjects, and analysed the relationship between those levels and HLA-DR genotypes or pathogenic bacteria levels using human saliva.

NOD/LtSz-scid (nonobese diabetic – severe combine immunodeficiency, NOD-SCID) mice grafted with human peripheral blood mononuclear cells (hu-PBMC) have been used as *in vivo* models for studying human lymphoid cells responses to human specific antigens [36–38]. This mouse strain supports levels of human cell grafting that are 5 to 10-fold greater than those obtained in C.B-17-Scid mice [36]. As a result, the hu-PBMC-NOD-SCID mouse model is employed for long-term *in vivo* analysis of immunoregulatary interactions between human lymphocyte activation and antigen. We also investigated immunogenicity of PAc (361–386) peptide using the hu-PBMC-NOD-SCID mouse model to clarify direct evidence for induction of the specific antibody in human immune systems. Our results may provide useful information for the prevention of dental caries as well as diagnosis of their potential risk in humans.

MATERIALS AND METHODS

Mice

NOD-SCID mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the National Institute of Infectious Diseases (NIID). Female mice at the age of 6–9 weeks were used in the present study. All experiments were performed in accordance with our institutional guidelines.

Human subjects

One hundred and fifty-one patients (60 males, age 37.6 ± 13·8; 91 females, age 35·0 ± 10·4; Overall age 36·6 ± 12·6 years old) of the Pacific Dental Clinic, Japan, participated in this study. Prior to the survey, the aim and details of the experiments were explained and consent was obtained from all subjects. The study was conducted according to the ethical guideline at our institution according to the Helsinki declaration. Dental examinations were conducted under artificial white light by trained dentists. According to WHO criteria [39], decayed teeth (DT), missing teeth (MT), and filled teeth (FT) (DMFT) scores were recorded along with findings of dental caries. Genetic (phenotypic) typing for HLA-*DRB1* was determined using a PCR-restriction fragment length polymorphism method by the Tissue Typing Department (BML, Tokyo, Japan) with samples from 96 of the subjects.

PAc peptide synthesis

The sequences of PAc (361–386) (NAKATYEAALKQYEAD LAAVKKANAA) and PAc (346–364) (AALTAENTAIK QRNENAKA) were derived from the sequence of the PAc gene from *S. mutans* MT8148, which corresponds to a portion of the A repeat, as described by Okahashi *et al.* [40]. The PAc (residue 361–386) peptide in the A-region possesses a multiple binding motif (L- -V-K- -A) to various HLA-DR molecules and the B-cell core epitope (- Y- - -L- -Y- - - -), which is used for recognizing the inhibiting antibody to *S. mutans*. The peptide was synthesized by a stepwise solid phase procedure at Asahi Techno Glass Co. Inc. (Tokyo, Japan). The synthesized peptide samples were subsequently purified by reversed-phase high-performance liquid chromatography (HPLC) on a TSK-GEL column (1 × 30 cm) (TOSO, Tokyo, Japan) with a 10% to 45% acetonitrile gradient in 0·1% TFA, and developed over 50 min at a flow rate of 5 ml/minute. Purity was determined as greater than 95% in each tube by HPLC analysis. To confirm the amino acid sequences of the synthetic peptides, several samples were randomly selected, and then analysed using a System 7300 Amino Acid Analyser (Beckman, NJ) and a Model 477 A Protein Sequencer (Applied Biosystems, Foster city, CA, USA).

Human saliva collection

Whole saliva from human subjects was stimulated by chewing paraffin gum and collected into ice-chilled sterile bottles over a period of 5 min, and clarified by centrifugation at 10 000× g for 10 min at 4°C. Saliva samples were also collected in plastic tubes and stored at −80°C, then defrosted just prior to measuring the antibody levels.

Bacteria counting

All bacteria counting was performed by the Laboratory of Bacteriology (BML). Saliva samples were gently shaken and inoculated onto Mitis-Salivarius agar (MTS, Nippon Becton Dickinson Co. Ltd, Tokyo, Japan) and Rogosa SL agar (Nippon Becton Dickinson Co. Ltd) using an EDDY JET spiral plating system (IUL, S.A., Torrent, Spain), to count total streptococci (tS) and lactobacilli (LB) organisms. Modified MTSB (MMTSB) was prepared by a classic modification of MTS agar plates containing 0·02 M bacitracin (MTSB, Sigma Chemical Co., St. Louis, MO), and used for detection and counting of mutans streptococci (mS) organisms. The MMTSB contained 20% sucrose (Wako, Tokyo, Japan), 2 μg/ml of gramicidin (Sigma), 10 μg/ml of nalidixic acid (Wako), 10 μg/ml of colistin sulphate (Wako), and 2 mg/ml of yeast extract (Becton Dickinson Sparks, MD), and is known to be extremely precise for the counting of mS colonies [41–44]. Following anaerobic inoculation for 48 h at 37°C, the colony-forming units (CFU) of every group were counted. Colonies of mS were identified by their characteristic appearance and the mS ratio was calculated as colony numbers of mS/colony numbers of tS × 100.

Injection of PAc (361–386) peptide to humanized mice

The immunization schedule was shown in Fig. 2a. Transplantation of hu-PBMC into NOD-SCID mice was performed using procedures and conditions described previously [45]. hu-PBMC were isolated from 400 ml of peripheral blood taken from a normal healthy volunteer by separation using Ficoll-Conrey (Immuno-Biological Laboratories, Gunma, Japan) density gradient centrifugation. The cells were washed 3 times in Hanks Balanced Salt Solution (HBSS) (Gibco Laboratories, Life Technologies, Paisley, UK) and adjusted to a concentration of $4{\cdot}0–8{\cdot}0 \times 10^7$/ml in HBSS. hu-PBMC suspensions were then administered intraperitoneally at 0·5 ml per mouse. Groups of 3–5 female mice from a single litter were grafted with PBMC from the donor and used in the experiments. Mice were irradiated (gamma irradiation, 2·5 Gy) from a ^{137}Cs source (Gamma cell 40, Atomic Energy of Canada Ltd, Kanata, Canada) 0–1 days before human cell transfer. On 1, 7 and 14 days after hu-PBMC transplantation, some of the hu-PBMC-NOD-SCID mice were administrated intraperitoneally with a mixture of 0·0 or 250·0 ng of hu-IL-4 (204-IL, R & D system Inc, Minneapolis, MN, USA) or IL-10 (MC/9, BioSource, Camarillo, CA, USA), with 0·0 and 30·0 ng of PAc (361–386) peptide in 300 μl of phosphate-buffered saline (PBS), pH 7·4. Seven days after hu-PBMC transplantation, the mice were immunized subcutaneously with 30·0 ng of PAc (361–386) peptide emulsified in Freund's complete adjuvant (Difco Laboratory, Detroit, MI, USA). One week later, the mice were boosted by a subcutaneous injection with and without the immunizing antigen at the same dose emulsified in Freund's incomplete adjuvant (Difco). Control mice without the immunizing antigen were injected consistently with 300 μl of PBS alone. One week after the last injection, sera and spleens were extracted for testing. Genotyping for HLA-*DRB1* in the spleen cells from hu-PBMC-NOD-SCID mice injected or not injected with the peptide was performed by the Tissue Typing Department of BML.

ELISA

For an enzyme-linked immunosorbent assay (ELISA), 96-well microtiter H-plates (Sumitomo Bakelite, Tokyo, Japan) were coated overnight at 4°C with 100 μl of PAc (361–386) peptide (concentration 20 μg/ml) or skim milk (as a control) in coating buffer at pH 9·6 for enumeration of the IgG specific to *S. mutans* [17]. The plates were washed with PBS containing 0·1% (v/v) Tween 20 (PBST) and blocked with 1% (wt/vol) skim milk in PBST for 1 h at 37°C. Excess skim milk was removed by washing 3 times with PBST, and then a 100 μl aliquot of a twofold serial dilution of saliva or sera from the inoculated hu-PBMC-NOD-SCID mice was added to the wells and the mixtures were incubated for 1 h at 37°C. The wells were then washed 5 times with PBST and further incubated for 1 h at 37°C with 100 μl of alkaline phosphatase-conjugated goat antihuman immunoglobulin A or G (both heavy and light chains) antibodies (Zymed Laboratories, South San Francisco, CA, USA). After 5 washes with PBST, bound antibodies were detected after the addition of 100 μl of 3 mg/ml para-nitrophenyl phosphate as a substrate and incubation for 90 min at 37°C. Absorbance at 405 nm (A_{405}) was measured with a microplate reader (Multiskan Bichromatic; Laboratory Japan, Tokyo, Japan). The ELISA-antibody titre was expressed as the reciprocal (Log_2) of the highest dilution giving an A_{405} of 0·1 above that of the control (skim milk) after 1 h of incubation with the substrate.

Dot blotting

To confirm the specificity of the anti-PAc(361–386) peptide antibody in human saliva, dot blot analysis was performed using bovine serum albumin (BSA) with BSA-conjugated PAc (361–386) peptide blotted onto the nitrocellulose. The nitrocellulose blots were incubated in human saliva and alkaline phosphate-conjugated goat polyclonal antibodies raised against the human IgA antibodies, and then exposed to the substrate.

Flow cytometry

Single cell suspensions of spleen cells were prepared by gently homogenizing the cells with ice-cold HBSS. Single cell suspensions of peritoneal cells were collected by washing the peritoneal cavity with an HBSS solution. All cell suspensions were washed once in ice-cold HBSS as described below. Spleen or peritoneal cells were stained with FITC- or PE-conjugated antihuman marker mAbs in PBS/1% BSA and washed with HBSS medium. At least 10^4–10^5 live spleen cells, including mouse and human lymphoid cells, were acquired in each run. For each mouse analysed, cells were also stained with mouse IgG conjugated to FITC and PE as an isotype control. Spleen or peritoneal cells from a non-transplanted NOD-SCID mouse were stained in parallel as an additional negative control. Fluorescence levels that excluded greater than 98% of the cells in the negative controls were considered to be positive and specific for human staining. The cells were fixed in a 3% formalin/HBSS solution and stored at 4°C until flow cytometric analysis. Samples gated on the forward light scatter (FSC) and side light scatter (SSC) were used to identify viable lymphocytes. Proportions of the major subsets were determined by single and quadrant analyses. Single cell suspensions were stained with the following antibodies: fluorescein isothiocyanate (FITC)-conjugated antimouse CD45 (30-F11), antihu-CD45 (H130), antihu-CD4 (RPA-T4), and phycoerythrin (PE)-conjugated antihu-CD8 (RPA-T8), each purchased from BD PharMingen (San Diego, CA, USA). The percentages of FITC and PE-positive cells were measured using a FACS with the CELLquest program (Beckton Dickinson, San Jose, CA, USA).

Statistical analysis

Allele frequencies in the human subjects were calculated by direct counting. Group comparisons of the levels of parameters were analysed by ANOVA. *P*-values of ≤0·05 were considered to be statistically significant.

RESULTS

Correlations between anti-PAc (361–386) peptide antibody titre and various parameters

The differences between female and male subjects for age, anti-PAc (361–386) peptide IgA (aPPA) titre in saliva, DMFT, LB, mS number, mS ratio, and tS number were investigated. DMFT ($15{\cdot}1 \pm 7{\cdot}0$) in females was significantly higher than in males ($12{\cdot}4 \pm 4{\cdot}8$, $P < 0{\cdot}05$), whereas there were no significant differences between the other parameters. The human subjects were divided into 4 groups: the no antibody group (anti-PAc (361–386) peptide antibody titre (a) ≤0·1), low group (>0·1 but ≤1), moderate group (>1 but ≤3) and high group (≥3), and the various parameters were compared within each (Table 1). Reactions to the peptide were determined by ELISA, and also confirmed by dot blot analysis using BSA-conjugated PAc (361–386) peptide and the control (BSA) (data not shown). Mean age

Table 1. Relationship between anti-Pac (361–386) peptide antibodies in saliva and various parameters

Groups	n	F:M	Age	DMFT	LB (×10⁸/ml)	mS (×10⁵/ml)	mS ratio (%)	tS (×10⁷/ml)
No antibody	20	11:9	44.8 ± 14.7	13.8 ± 4.8	1.3 ± 3.4	4.2 ± 7.7	0.7 ± 1.2	4.0 ± 3.4
Low	32	18:14	38.5 ± 12.9	14.4 ± 5.5	2.7 ± 6.6	2.2 ± 3.7	0.8 ± 1.6	4.2 ± 3.5
Moderate	58	37:21	35.2 ± 11.5	13.7 ± 5.5	3.0 ± 8.3	3.0 ± 6.2	0.9 ± 1.5	3.6 ± 3.7
High	41	25:16	33.0 ± 11.1	14.3 ± 8.5	2.4 ± 4.1	1.0 ± 2.6	0.3 ± 0.5	4.3 ± 5.7
Total	151	91:60	36.6 ± 12.6	14.0 ± 6.4	2.5 ± 6.3	2.4 ± 5.3	0.7 ± 1.3	3.9 ± 4.2

Anti-Pac (361–386) peptide antibody titre in saliva: No antibody group ≤0.1; Low >0.1 and ≤1.0; Moderate >1.0 and ≤3.0; High >3.0. F, female; M, male; n, no. of subjects. Significant differences between each genotype $^{*}P < 0.05$, $^{**}P < 0.01$

(44·8 ± 14·7 years) was significantly higher in the no antibody group as compared to the moderate (35·2 ± 11·5 years) and high (33·0 ± 11·1 years) groups ($P < 0·01$). The number of mS (1·0 ± 2·6) and mS ratio (0·3 ± 0·5) in the high group were significantly lower than those in the no antibody (4·2 ± 7·7) and moderate (0·9 ± 1·5) groups ($P < 0·05$). There were no significant differences between DMFT, LB and tS concentration, and aPPA. The PAc (346–364) peptide contains the B cell epitope in humans [16] and was used as a control antigen. There were no observable differences between the various parameters and anti-PAc (346–364) peptide IgA antibodies in many of the saliva samples ($n = 70$) (data not shown).

Age, mS number, and mS ratio were compared between the 4 groups, and between females and males (Fig. 1). The antibody titre showed a significantly negative correlation with age in males, while mS number was significantly higher in the no antibody group as compared to the moderate and high groups among females, and higher in the moderate as compared to the high group among males ($P < 0·05$). However, there were no significant differences in mS ratio between females and males in all groups.

Correlations between DRB1 genotypes and anti-PAc (361–386) peptide antibodies

The associations between various *DRB1* genes, and the titres and bacterial parameters, as seen by ANOVA, are shown in Table 2. The aPPA titre was significantly lower in the mixed genotypes of homozygous *DRB1*0405* and *1502*, and *DRB1*0405/*1502* than

Table 2. Correlations with *DRB1*0405, 1502, 1501, 0901* or *0101* to various parameters

DRB1	n	F:M	Age	Titer	DMFT	LB (×10⁵/ml)	mS (×10⁵/ml)	mS ratio (%)	tS (×10⁷/ml)
0405 1502 homo *0405/1502*	9	5:4	38.4 ± 11.5	0.4 ± 0.5	12.0 ± 4.0	1.3 ± 2.2	1.2 ± 1.6	0.4 ± 0.9	1.6 ± 1.5
0405 hetero	17	13:4	38.9 ± 13.4	2.6 ± 1.3	13.2 ± 5.2	1.0 ± 3.1	1.6 ± 2.0	0.7 ± 0.9	3.7 ± 2.6
1502 hetero	18	15:3	39.9 ± 14.3	2.2 ± 1.5	13.9 ± 5.6	1.0 ± 2.6	2.9 ± 6.4	0.9 ± 1.5	3.4 ± 3.1
1501	11	5:6	37.0 ± 11.9	2.7 ± 1.0	14.2 ± 6.9	0.1 ± 0.1	0.7 ± 1.3	0.2 ± 0.0	6.4 ± 4.2
0901	25	16:9	40.4 ± 16.0	2.1 ± 1.5	14.5 ± 6.1	3.4 ± 8.5	2.8 ± 4.3	0.9 ± 1.1	4.9 ± 3.4
0101	15	8:7	34.9 ± 12.6	1.5 ± 1.1	13.7 ± 5.5	2.2 ± 5.2	1.2 ± 2.5	0.3 ± 0.4	4.0 ± 3.8
Others	21	15:6	44.7 ± 15.7	1.6 ± 2.2	13.9 ± 4.2	1.1 ± 3.0	3.4 ± 6.1	1.1 ± 1.7	3.9 ± 2.8

0405, 1502 homo, *0405/1502*: Subject group expressing HLA- *DRB1*0405/0405, 1502/1502* or *0405/1502*. *0405* hetero: Subject group expressing HLA- *DRB1*0405*/others. *1502* hetero: Subject group expressing HLA- *DRB1*1502*/others. *1501* hetero: Subject group expressing HLA- *DRB1*1501*/others. *0901*: Subject group expressing HLA- *DRB1*0901* allele. *0101*: Subject group expressing HLA- *DRB1*0101* allele. Others: Subject group expressing HLA- *DRB1*1502, 1501, 0405, 0901* and *0101* allele. Significant differences between each group $^{*}P < 0.05$, $^{**}P < 0.01$; numbers shown on square brackets are *P*-value without statistical significance

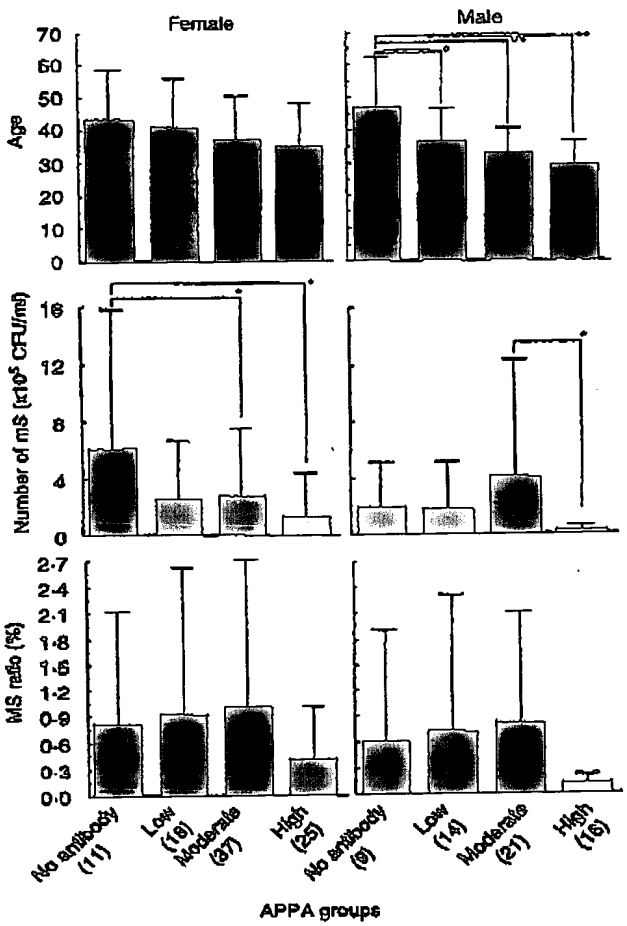

Fig. 1. Relationships between age, number of mS organisms, mS ratio, and levels of a-PPA in female and male subjects. Measurement of mS number and distribution of a-PPA titre in 4 groups are described in Materials and Methods. Results are expressed as mean ± SD of each parameter. ( ): Number of subjects in each age group. Asterisks denote significantly different relative antibody level ($^{*}P < 0.05$, $^{**}P < 0.01$).

heterozygous *DRB1*0405, DRB1*1502, DRB1*1501,* and others. The number of tS was also lower in the mixed genotype group than *DRB1*1501, 0901, 0101,* and others. In contrast, the titre was higher in heterozygous *DRB1*1501* than the mixed genotype, *DRB1*0101,* and others, while LB and mS ratio were significantly lower than in *DRB1*0901* and others. Further, the titre as well as tS number and LB were higher in the *DRB1*0901* than the mixed genotypes and *DRB1*1501*. There were no significant differences between various DRB1 groups in Age, DMFT and mS.

Production of hu-anti-PAc (361–386) peptide IgG antibodies in mice

To establish a small animal model for production of hu-IgG antibodies to PAc (361–386) peptide, we grafted hu-CD45^{+}, -CD4^{+} and -CD8^{+} cells and injected the PAc peptide into NOD-SCID mice, after which we analysed production of the hu-anti-PAc (361–386) peptide IgG antibody (aPPG) in the those hu-PBMC-NOD-SCID mice. IL-4 and IL-10 are pleiotrepic cytokines produced by activated Th2 cells [46,47] that have been identified as important regulators for B cell development [48]. Further, we investigated whether IL-4 or IL-10 had an effect to regulate the production of aPPG. Prior to the analysis for aPPG, significant proportions of hu-CD45^{+}, -CD4^{+}CD8^{-}, and -CD4^{-}CD8^{+} cells in the spleen and peritoneal cells were also detected by FACS analysis to determine the grafting efficacy of the hu-cells into the mice (data not shown). The production of aPPG was up-regulated by intraperitoneal administration of the peptide (30 ng/mouse) without cytokines in 7 of 9 mice expressing different heterozygous DRB1 genotypes and 1 mouse expressing homozygous *DRB1*0405*, in contrast to the nonimmunized mice (Fig. 2b). In addition, co-administration of IL-4 with the peptide showed positive effects for increasing aPPG (Fig. 2c), whereas coadministration of IL-10 inhibited the increase in 4 of 6 mice expressing different heterozygous *DRB1* genotypes and 1 mouse expressing homozygous *DRB1*0405* (Fig. 2d).

DISCUSSION

There has been increasing interest in recent years in the establishment of a PAc peptide antigen, and studies of a candidate antigen, PAc (361–377) peptide, as well as T and B-cell epitopes that induce an inhibiting antibody to interaction with salivary components and colonization of *S. mutans* on the rat's tooth surfaces and the multiple agretope (L--V-K--A) that are restricted by various HLA-DR genotypes have been reported [17–19,21,23]. The spread peptide, PAc (361–386) peptide to the C-terminal of PAc (361–377) peptide, includes a multiple agretope. The PAc (361–377) peptide has been shown to induce specific antibodies to mutans streptococci (*S. mutans* and *S. sobrinus*), however, not other streptococci in mice, because the peptide possesses a high homologous amino acid sequence between *S. mutans* and *S. sobrinus* [18,20,21]. As a result, the PAc (361–386) peptide is considered to be a candidate antigen for induction of the antibody that specifically inhibits colonization of *S. mutans* and *S. sobrinus* in humans.

S. mutans is a pathogen of dental caries, infecting the oral cavity of almost all humans. The PBMC from subjects used in the present study showed positive serum anti PAc(361–386) peptide antibody level in hu-PBMC-NOD-SCID mice injected with control-PBS and were thus sensitized to *S. mutans* antigens. Accordingly, it is speculated that, in the hu-PBMC-NOD-SCID mice to whom *S. mutans*-sensitized PBMC had been transplanted, the specific antibodies produced might have resulted from secondary responses to the immunization with the PAc(361–386) peptide. Consequently, PAc (361–386) peptide was confirmed as an ideal peptide antigen for induction of the antibody in humans by ELISA and the NOD-SCID mouse system. Recent studies involving immunization with synthetic peptides and fusion proteins with PAc from the catalytic and glucan-binding regions of glucosyltransferase (GTF) have shown a reduction in the level of smooth surface caries in both active and passive immunized rats following infection with *S. mutans* or *S. sobrinus* [15,49]. Several GTF and PAc peptides speculated to have high binding characteristics to MHC class II have also been studied for their immunogenicity in rats and mice [50,51], and the binding motifs of GTF to MHC class II have been reported as well [22]. However, the antigenicities of these peptides have not been investigated in a

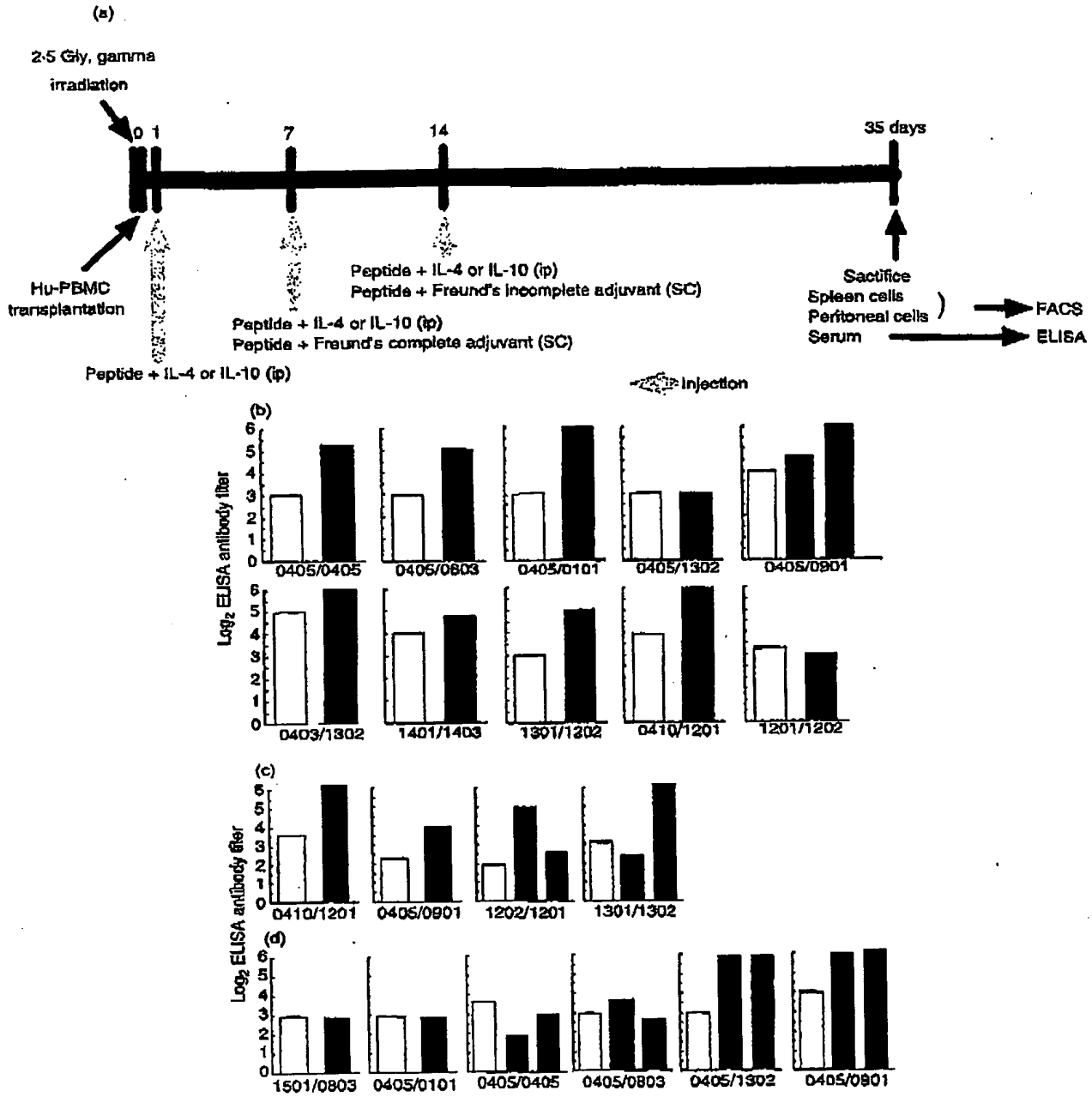

Fig. 2. Production of a-PPG in hu-PBMC-NOD-SCID mice following immunization with PAc (361–386) peptide. The immunization schedule was shown in (a). The peptide (■) in PBS (b), IL-4 (c), or IL-10 (d) was injected into 1 or 2 NOD-SCID mice grafted with hu-PBMC expressing the heterozygous or homozygous HLA-*DRB1* genotype from a single donor. The peptide immunization procedure is described in Materials and Methods. The bar graph shows the Log2 ELISA antibody titre in individual injected mouse serum samples. As a control, a PBS injection without the peptide (□) was performed and the peptide immunogenicity was compared with the control in the production of a-PPG in mice sera.

human immune system. The present study is the first to show that the PAc (361–386) peptide is a unique antigen for the recognition and induction of inhibiting antibodies to mutans streptococci in the human immune system. Our results may provide useful information for the construction of peptide-based vaccines using various epitopes in PAc and GTF to prevent dental caries.

Our findings suggest that production of the anti-PAc (361–386) peptide antibody is regulated by ageing, HLA-DR genotype, and cytokines, as the antibody titre was found to have a negative correlation with ageing, with a significant correlation in males however, not in females, who might have had a menopausal disorder or pregnancy at some time (Fig. 1). Optimum level of the antibody titre was also required for the decrease of mutans streptococci in saliva. Wallengren *et al.* [52] indicated that the level of salivary antibody response differs among genetically different individuals. Some investigators have also reported a relationship between HLA and caries susceptibility [53–55], as well as associations between HLA class II genes and mS and/or LB [23,27,52,56]. Further, Acton *et al.* demonstrated that DR-4 might have a part in controlling dental caries and that *DRB1*-4 allele frequencies in African-American women were positively associated with *S. mutans* level [56]. In a recent study, Wallengren *et al.* [35] found lower salivary IgA activity in response to *S. mutans* in tests with *DRB1*0401* and *0404*, while Ozawa *et al.* showed that there was no association between DR-4 (*DRB1*0405*) and mutans streptococci or lactobacilli [57]. In the present study, homozygous *DRB1*0405* in the mixed genotypes showed a negative association with production level of aPPA and numbers of tS bacteria in saliva, while heterozygous *DRB1*0405* showed a positive association with the production level of aPPA, however, not with other bacterial parameters. Therefore, the *DRB1*-4 allele may respond to an antigen presenting molecule of *S mutans*. *DRB1*0405* showed poor reactivity to PAc (361–386) peptide in its homozygous expression and did not disturb the susceptibility of another *DRB1** allele to the peptide in an individual with 2 MHC genotypes. Previous reports as well as our studies of various subject groups have revealed unique features regarding the DR4 association, and the present findings may suggest involvement of the *DRB1*0405* allele and others in the aetiology of oral streptococci.

In addition to *DRB1*0405*, homozygous and heterozygous *DRB1*1502* in mixed genotypes showed susceptibility similar to *DRB1*0405*, as the mixed group of homozygous *DRB1*0405* and *1502*, and heterozygous *DRB1*0405/1502* were correlated with lower levels of tS therefore they may have an association with the regulation of oral flora. Our data also indicate that heterozygosity or homozygosity of *DRB1*0901* increases predisposition to a high frequency of LB in saliva, and that *DRB1*1501* positive status in individuals produces aPPA and eliminates the susceptibility conferred by other HLA-DR *DRB1* genotypes to colonization by mS and LB. A negative association with the haplotype that includes the *DRB1*1501* allele was also reported in *Helicobacter pylori* related diseases [58], while patients with the *DRB1*1501/DQB1*0602* haplotype showed significantly reduced responses and were less likely to develop severe systemic diseases caused by group A streptococcal infections [59]. These results indicate that the *DRB1*1501* allele may be involved with resistance to infectious diseases occurring in the upper alimentary region by employing mucosal immunity.

Based on our findings, we propose that host immunogenic factors involved in regulating PAc responses may have an influence on the severity of mutans streptococci colonization. Our data also suggest that this effect is mediated through differential presentation of streptococcal PAc by distinct class II alleles, resulting in significant differences in the magnitude of mutans streptococci biofilm formation. The effects of class II allelic variation on the induction of inhibiting antibodies may also be regulated by polymorphisms of other host immunogenetic factors such as cytokines. This wide scope of regulators provides an intriguing model for investigation of the role of host-biofilm formation and understanding of the underlying mechanism of these genetic associations. However, there were no significant differences between DMFT and the other parameters, except gender, in the present study. In adult individuals, it may be difficult to clarify the associations of DMFT with microbial parameters or antibody titre, as not only dental caries but also periodontal diseases have an association with missing teeth, and can reveal past dental status [60]. Therefore, a definitive discussion regarding the relationship between the antibody titre and DMFT requires further investigation in young population.

In general, oral biofilm exhibits significant resistance to antimicrobial agents and is capable of a strong physiological response to agent-caused stress. The differential agent sensitivity of biofilm and dispersed biofilm cells indicate that its physical structure enhances normal cellular systems for growth, which are dependent on the nutritional status of the organism [61]. The adherence of planktonic cells to a surface structure is inhibited by agents such as anti-PAc (361–386) peptide antibody and may not induce the appearance of biofilm, therefore, the present findings indicate that immunological elements of the host defense system operate in cooperation with each another. The protective features of this antibody may make it possible to design a multiepitope caries vaccine to be given to individuals expressing various MHC class II types. In the future, a mucosal adjuvant such as mutant cholera toxin [62] may be a powerful means to safely elevate the level of antibody in a peptide vaccination. In addition, regulators of antibody induction may also be used as indicators of dental caries risk for development of a diagnostic method.

ACKNOWLEDGEMENTS

This work was supported in part by a grant-in aid for Development Scientific Research (15390571) from the Ministry of Education, Science, and Culture of Japan, and by a grant from the Japan Health Science Foundation to H.S.

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